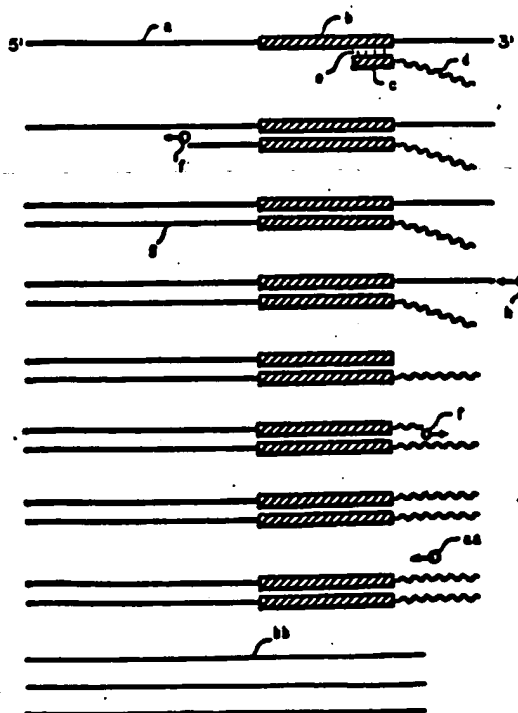




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>4</sup>:</b>  <b>C12Q 1/68</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 89/06700</b>  <b>(43) International Publication Date:</b> 27 July 1989 (27.07.89)
<b>(21) International Application Number:</b> PCT/US89/00120 <b>(22) International Filing Date:</b> 12 January 1989 (12.01.89)  <b>(31) Priority Application Number:</b> 146,462 <b>(32) Priority Date:</b> 21 January 1988 (21.01.88) <b>(33) Priority Country:</b> US  <b>(71) Applicant:</b> GENENTECH, INC. [US/US]: 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).  <b>(72) Inventors:</b> MILLER, Harvey, I. ; 225 Linda Lane, Pleasant Hill, CA 94523 (US). JOHNSTON, Sean ; 3047 Emmerson Street, Palo Alto, CA 94306 (US).  <b>(74) Agents:</b> HENSLEY, Max, D. et al.; Genentech, Inc., Legal Department, 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (Euro- pean patent), CH (European patent), DE (Euro- pean patent), DK, FR (European patent), GB (Euro- pean patent), IT (European patent), JP, LU (Euro- pean patent), NL (European patent), NO, SE (Euro- pean patent).  Published <i>With international search report.</i> <i>With amended claims and statement.</i>  <b>Date of publication of the amended claims and statement:</b> 24 August 1989 (24.08.89)
<b>(54) Title: AMPLIFICATION AND DETECTION OF NUCLEIC ACID SEQUENCES</b>		
<b>(57) Abstract</b>  The present invention is directed to improved methods for assaying specific nucleic acid sequences in a test sample and the reagents for carrying out the methods. In the general, the methods of the invention involve the synthesis of a double-stranded nucleic acid containing the nucleic acid sequence to be detected and a promoter, the synthesis of a multiplicity of RNA transcripts under the control of the promoter, and the detection of the specific RNA transcripts produced.		



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IE	Ireland	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

## AMENDED CLAIMS

[received by the International Bureau on 28 July 1989 (28.07.89);  
original claims 1-30 and 36 cancelled; new claims 37-48 added (7 pages)]

31. A method for the detection of a specific nucleic acid sequence in a test sample containing single-stranded DNA which comprises:

5

(a) contacting the test sample with an oligonucleotide promoter-primer, comprising a promoter for a dedicated RNA polymerase ligated to a primer, under conditions that permit hybridization of the promoter-primer to the nucleic acid sequence to be detected;

10

(b) contacting the test sample with a DNA polymerase, such that a double-stranded nucleic acid is synthesized, comprising the nucleic acid sequence to be detected and the promoter of the promoter-primer;

15

(c) contacting the product of step (b) with a dedicated RNA polymerase capable of recognizing the promoter of the oligonucleotide promoter-primer, whereby a multiplicity of RNA transcripts of the nucleic acid sequence to be detected are synthesized by the RNA polymerase under the control of said promoter;

20

(d) determining the presence of RNA transcripts synthesized in step (c); and

25

(e) correlating the presence of the RNA transcripts with the presence of the nucleic acid sequence to be detected.

30

32. A method for the detection of a specific nucleic acid sequence in a test sample containing RNA or single-stranded DNA which comprises:

- 5 (a) contacting the test sample with an oligonucleotide promoter-primer, comprising a promoter for a dedicated RNA polymerase ligated to a primer, under conditions that permit hybridization of the promoter-primer to the specific nucleic acid sequence to be detected;
- 10 (b) contacting the test sample with reverse transcriptase such that a promoter-primer DNA extension product is synthesized, wherein the template for synthesis of the promoter-primer DNA extension product is the test sample RNA or single-stranded DNA to which the promoter-primer has hybridized in step (a);
- 15 (c) treating the product of step (b) under denaturing conditions to separate the promoter-primer DNA extension product from its template;
- 20 (d) contacting the single-stranded nucleic acid produced in step (c) with an oligonucleotide secondary primer, wherein said secondary primer is selected to be homologous to all or part of the nucleic acid sequence to be detected and not complementary to the promoter-primer used in step (a), under conditions that permit hybridization of the secondary primer to the promoter-primer DNA extension product;
- 25 (e) contacting the product of step (d) with a DNA polymerase or reverse transcriptase, such that a secondary primer DNA extension product is synthesized, wherein the template for synthesis of the secondary promoter DNA extension product is the promoter-primer DNA extension product;
- 30 (f) contacting the product of step (e) with a dedicated RNA polymerase capable of recognizing the promoter of the oligonucleotide promoter-primer, whereby a multiplicity of RNA

transcripts of the nucleic acid sequence to be detected are synthesized under the control of said promoter; and

(g) correlating the presence of the RNA transcripts with the presence of the nucleic acid sequence to be detected.

33. A method for the detection of a specific nucleic acid sequence in a test sample containing RNA or single-stranded DNA which comprises:

(a) contacting the test sample with an oligonucleotide secondary primer, under conditions that permit hybridization of the secondary primer to the specific nucleic acid sequence to be detected;

(b) contacting the test sample with reverse transcriptase such that a secondary primer DNA extension product is synthesized, wherein the template for synthesis of the secondary primer DNA extension product is the test sample RNA or single-stranded DNA to which the secondary primer has hybridized in step (a);

(c) treating the product of step (b) under denaturing conditions to separate the secondary primer DNA extension product from its template;

(d) contacting the single-stranded nucleic acid produced in step (c) with an oligonucleotide promoter-primer, comprising a promoter for a dedicated RNA polymerase ligated to a primer, wherein said promoter-primer is selected to be homologous to all or part of the nucleic acid sequence to be detected and not complementary to the secondary primer used in step (a), under conditions that permit hybridization of the promoter-primer to the secondary primer DNA extension product;

(e) contacting the product of step (d) with a DNA polymerase such that a promoter-primer DNA extension product is synthesized, wherein the template for synthesis of the promoter-primer DNA extension product is the secondary primer DNA extension product;

(f) contacting the product of step (e) with a dedicated RNA polymerase capable of recognizing the promoter of the oligonucleotide promoter-primer, whereby a multiplicity of RNA transcripts of the nucleic acid sequence to be detected are synthesized under the control of said promoter; and

(g) correlating the presence of said RNA transcripts with the presence of the nucleic acid sequence to be detected.

34. A method for the detection of a specific nucleic acid sequence in a test sample containing single-stranded DNA which comprises:

(a) contacting the test sample with an oligonucleotide secondary primer, under conditions that permit hybridization of the secondary primer to the specific nucleic acid sequence to be detected;

(b) contacting the test sample with a DNA polymerase such that a secondary primer DNA extension product is synthesized, wherein the template for synthesis of the secondary primer DNA extension product is the test sample RNA or single-stranded DNA to which the secondary primer has hybridized in step (a);

(c) treating the product of step (b) under denaturing conditions to separate the secondary primer DNA extension product from its template;

(d) contacting the single-stranded nucleic acid produced in step (c) with an oligonucleotide promoter-primer, comprising a promoter for a dedicated RNA polymerase ligated to a primer, wherein said promoter-primer is selected to be homologous to all or part of the nucleic acid sequence to be detected and not complementary to the secondary primer used in step (a), under conditions that permit hybridization of the promoter-primer to the secondary primer DNA extension product;

10

(e) contacting the product of step (d) with a DNA polymerase such that a promoter-primer DNA extension product is synthesized, wherein the template for synthesis of the promoter-primer DNA extension product is the secondary primer DNA extension product;

15

(f) contacting the product of step (e) with a dedicated RNA polymerase capable of recognizing the promoter of the oligonucleotide promoter-primer, whereby a multiplicity of RNA transcripts of the nucleic acid sequence to be detected are synthesized under the control of said promoter; and

20

(g) correlating the presence of said RNA transcripts with the presence of the nucleic acid sequence to be detected.

25

35. A kit for use in the detection of a nucleic acid sequence in a test sample containing an oligonucleotide promoter-primer and an oligonucleotide probe, wherein the promoter-primer is capable of hybridizing to the test sample nucleic acid sequence and the oligonucleotide probe is homologous to all or part of the nucleic acid sequence to be detected.

30

37. A method for preparing a double-stranded nucleic acid which

includes a promoter operably linked to a sequence to be detected, which method comprises:

(a) providing an oligonucleotide promoter-primer;

(b) contacting said promoter-primer with a nucleic acid comprising the sequence to be detected under conditions that permit hybridization of the promoter-primer to the nucleic acid sequence to be detected;

(c) synthesizing an extension product from the 3' end of the promoter-primer, which extension product is complementary to the nucleic acid sequence to be detected;

(d) contacting the product of step (c) with an agent possessing 3' - 5' exonuclease activity; and

(e) synthesizing an extension product from the 3' end of the sequence to be detected, which extension product is complementary to the promoter of the promoter-primer.

38. The method of claim 37 wherein the promoter-primer of step (a) is the only primer utilized.

39. The method of claim 37 wherein the nucleic acid comprising the sequence to be detected is digested with a restriction enzyme before or during step (b).

40. The method of claims 37 or 38 wherein the synthesis of the extension products of steps (c) and (e) is accomplished using an enzyme selected from the group consisting of *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, and T4 DNA polymerase.

41. The method of claims 37 or 38 wherein the agent of step (d) is an enzyme selected from the group consisting of *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, and T4 DNA polymerase.



42. The method of claims 37 or 38 wherein the synthesis of the extension products first p (c) and (e) is accomplished using the same agent as that utilized in step (d).
- 5 43. The method of claims 37 or 38 wherein steps (c), (d), and (e) are carried out simultaneously in the same reaction vessel.
- 10 44. A method useful for the detection of a specific nucleic acid sequence in a test sample containing nucleic acid, comprising the synthesis of a plurality of RNA transcripts from a double-stranded nucleic acid prepared according to the method of claim 37 or claim 38, wherein each RNA transcript comprises an RNA sequence corresponding to the specific nucleic acid sequence to be detected, and determining the presence of said RNA sequence.
- 15 45. The method of claim 44 wherein the plurality of RNA transcripts is synthesized using T7 RNA polymerase.
- 20 46. The method of claim 44 wherein the plurality of RNA transcripts is synthesized using SP6 RNA polymerase.
- 25 47. The method of claim 44 wherein the presence of said RNA sequence is determined by contacting the RNA transcripts under hybridizing conditions with an oligonucleotide probe selected to hybridize with a predetermined sequence within the RNA transcripts.
- 30 48. The method of claim 47 wherein the RNA transcripts are immobilized on a solid support.

5

## STATEMENT UNDER ARTICLE 19

10

15           Claims 1-30 and 36 have been cancelled. In their place is  
added new claims 37-48. The basis for the additional claims is  
found in the original claims and in the specification, as follows:

	<u>Claim</u>	<u>Basis</u>
20	37	Specification: p.14, line 28- p.16, line 13; Example 1; Fig.1.
25	38	Specification: p.13, line 26- p.14, line 4; Example 1.
	39	Original claim 9.
30	40	Original claim 11; Specification: p.16, lines 5-9.
	41	Specification: p.16, lines 5-9.
35	42	Specification: p.15, line 34- p.16, line 5.
	43	Specification: p.15, line 34- p.16, line 5.

	44	Original claim 1; Specificati n: p.5, lines 24-34; p.16, line 15- p.18, line 27; Fig.1.
5	45	Original claim 7; p.9, lines 1- 5; Example 1.
10	46	Original claim 8; p. 9, lines 1- 5.
	47	Original claim 21; Specification: p.17, lines 9-14; p.18, lines 4- 18.
15	48	Original claim 22; Specificati n: p.18, lines 16-18.
20		
25		
30		



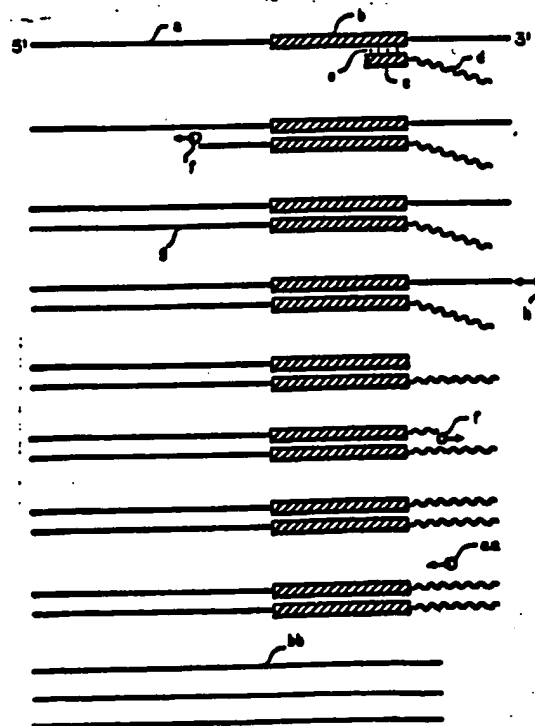
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>4</sup> :  C12Q 1/68	A1	(11) International Publication Number: WO 89/ 06700 (43) International Publication Date: 27 July 1989 (27.07.89)
<p>(21) International Application Number: PCT/US89/00120</p> <p>(22) International Filing Date: 12 January 1989 (12.01.89)</p> <p>(31) Priority Application Number: 146,462</p> <p>(32) Priority Date: 21 January 1988 (21.01.88)</p> <p>(33) Priority Country: US</p> <p>(71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).</p> <p>(72) Inventors: MILLER, Harvey, I. ; 225 Linda Lane, Pleasant Hill, CA 94523 (US). JOHNSTON, Sean ; 3047 Emmerson Street, Palo Alto, CA 94306 (US).</p> <p>(74) Agents: HENSLEY, Max, D. et al.; Genentech, Inc., Legal Department, 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).</p>		<p>(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).</p> <p><b>Published</b> With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>

## (54) Title: AMPLIFICATION AND DETECTION OF NUCLEIC ACID SEQUENCES

## (57) Abstract

The present invention is directed to improved methods for assaying specific nucleic acid sequences in a test sample and the reagents for carrying out the methods. In the general, the methods of the invention involve the synthesis of a double-stranded nucleic acid containing the nucleic acid sequence to be detected and a promoter, the synthesis of a multiplicity of RNA transcripts under the control of the promoter, and the detection of the specific RNA transcripts produced.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

-1-

## AMPLIFICATION AND DETECTION OF NUCLEIC ACID SEQUENCES

Background of the Invention

5       Recent developments in the general field of molecular biology have made possible the detection of specific nucleic acid sequences of clinical and commercial importance. Analysis of the nucleic acid sequences of various human genes, for example, has revealed unique sequence alterations that are associated with specific  
10       diseases. Likewise, sequences have been identified within the genome of various pathogens which uniquely characterize each organism, and distinguish them from even closely related species. The availability of such sequence information has made possible the diagnosis of diseases at the genetic level.

15       The most common method for the detection of a specific nucleic acid sequence is hybridization. This method takes advantage of the ability of a nucleic acid sequence to form a stable noncovalent complex with a complementary nucleic acid sequence. In order to  
20       determine whether a specific nucleic acid sequence is present within a test sample, a complementary nucleic acid probe is prepared, labeled with a detectable chemical modification, and then added to the test sample. If the sequence to be detected is present, the labeled probe will become hybridized to it, with the  
25       detectable label providing the means for determining in a known manner whether hybridization has occurred, and to what extent.

30       The principal limitation on the use of present hybridization methods for the determination of a nucleic acid in a test sample is that they are not sensitive enough and therefore require a relatively large amount of sample to accurately verify the presence of a specific nucleic acid sequence. This limitation presents significant practical difficulties in a clinical setting because of the limited quantity of sample that is typically available for  
35       analysis. Consequently, much attention has been focused on

-2-

developing methods for improving the sensitivity of the hybridization method for detecting a specific nucleic acid sequence in a test sample and thereby expanding its utility in the diagnostic arts.

5

One general approach to improving the sensitivity of the hybridization method has been to increase the signal generated by the hybridization probe. Methods have been described, for example, for preparing nucleic acid probes labeled to high specific activity with radioactive labels, either by nick-translation (Rigby, et al., 10 1977, J. Mol. Biol. 113:237) or by SP6 transcription (Melton, et al., 1984, Nuc. Acids Res. 12:7035).

15

Schneider, et al., PCT Pat. App. WO 87/03622, describe the use of multiple signal-generating secondary probes, each capable of binding to a primary probe which has hybridized to a DNA or RNA sequence of interest, as a means for amplifying the signal generated by the hybridization event.

20

Chu, et al., PCT Pat. App. WO 87/06270 describe the use of "replicative RNA", alone or in conjunction with an affinity molecule, as a nucleic acid hybridization probe, and the use of an RNA-dependant RNA polymerase to replicate, and thereby increase the signal generated by, the bound probe RNA.

25

Rodland, et al., U.S. Pat. No. 4,647,529, teach the use of thionucleotides, which when incorporated into a hybridization probe increase the amount of binding between the probe and the nucleic acid sequence to be detected.

30

In contrast to the substantial efforts that have been made to increase the sensitivity of probe detection methods, relatively little research has been devoted to methods of amplifying the nucleic acid sequence to be detected in a test sample so that it is

-3-

produced in sufficient quantities to be detected by currently available hybridization methods.

5 Mullis, et al., U.S. Pat. No. 4,683,195, teach a process for amplifying a specific nucleic acid sequence in a test sample by the use of two oligonucleotide primers, and the synthesis of primer extension products. The extension product of one primer, when hybridized to the other primer, becomes a template for production of the desired specific nucleic acid sequence, and vice versa, and  
10 this process is repeated as often as necessary to produce the desired amount of the specific sequence.

Mullis, et al. also teach the use of this amplification method to prepare large quantities of a recombinant nucleic acid, using  
15 primers to which a non-complementary sequence is ligated. Mullis, et al. disclose ligating a nucleotide sequence for a promoter, linker, or coding sequence to one or both primers, whereby the non-complementary sequence is amplified together with the test sample nucleic acid sequence. In this manner, a large quantity of a  
20 recombinant nucleic acid is produced, consisting of an existing test sample nucleic acid sequence ligated to a selected exogenous nucleic acid sequence.

The essence of the Mullis, et al. process, therefore, is the  
25 amplification of a desired nucleic acid sequence in the form of complementary primer extension products. Because each primer extension product formed serves as the template for synthesis of yet another primer extension product, multiple repetitions of the Mullis, et al. primer extension procedure theoretically should  
30 result in the accumulation of the desired sequence at an exponential rate relative to the number of reaction cycles. In contrast, by-products formed by primer hybridizations other than those intended are not expected to be self-catalytic, and should therefore accumulate at a linear rate.

35



-4-

5 The specificity and accuracy of the Mullis, et al. process is thus seen to be strictly dependent upon the substantially increased rate of synthesis of the desired nucleic acid sequence relative to that of by-products. In practice, however, it is observed that the accumulation of by-products may proceed at a much greater rate than that predicted by theoretical calculations. Mullis et al., for example, disclose the use of their process to amplify a portion of the human beta-hemoglobin gene which is present in a sample containing human genomic DNA, and find that due to amplification of other sequences within the genome, only 1% of the final resulting population of amplified sequences correspond to the desired sequence.

15 To the extent that the Mullis, et al. process results in the amplification of nucleic acid sequences other than the one desired, the method assays a greater amount of the desired nucleic acid sequence than is actually present in the test sample, and produces false-positive results. It is one object of the present invention, therefore, to provide a method for specifically and quantitatively amplifying a desired nucleic acid sequence in a test sample, thereby improving the sensitivity of detection of the desired sequence, while avoiding the problems of false-positive and otherwise inaccurate results inherent in the existing technology.

25 Furthermore, the Mullis, et al. process contemplates multiple cycles of primer hybridization and primer extension synthesis, resulting in burdensome labor or automation costs. Automation of the Mullis, et al. process not only necessitates the use of specialized equipment but also the use of a specialized reagent, a heat-stable DNA polymerase, and reaction conditions tailored to each analyte test system so that the primer hybridization and primer extension synthesis steps may be conducted continuously. It is thus a further object of the present invention to provide a method for detecting a specific nucleic acid sequence in a test sample which is rapid and simple to perform without costly,

-5-

requiring various reaction steps, specialized equipment and reagents, and  
burdensome requirements for changing reaction conditions for each  
different test sample.

5     Summary of the Invention

The objects of this invention are accomplished by a method  
which comprises:

- (a) synthesizing a double-stranded nucleic acid which  
includes the sequence to be detected and a promoter;
- 10     (b) synthesizing a multiplicity of RNA transcripts from  
said double-stranded nucleic acid under the control of  
said promoter;
- (c) determining the presence of RNA transcripts produced  
in step (b); and
- 15     (d) correlating the presence of said RNA transcripts with  
the presence of the nucleic acid sequence to be detected.

In a further embodiment, the objects are accomplished by a  
method wherein the nucleic acid of step (a) is obtained by the  
20     steps comprising:

- (1) providing an oligonucleotide promoter-primer  
comprising a promoter ligated to a primer;
- (2) contacting the test sample with the promoter-primer  
under conditions that permit the hybridization of the  
25     promoter-primer to the nucleic acid sequence to be  
detected; and
- (3) synthesizing an extension product from the promoter-  
primer which is complementary to the nucleic acid  
sequence to be detected, using as a template the test  
30     sample nucleic acid to which the promoter-primer has  
hybridized in step (2).

In an additional embodiment, the objects are accomplished by a  
method wherein the nucleic acid of step (a) is obtained by the  
35     steps comprising:

-6-

- 5 (1) providing an oligonucleotide promoter-primer, comprising a promoter ligated to a primer, and an oligonucleotide secondary primer, wherein said primers are not complementary to one another, and are selected such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer;
- 10 (2) contacting the test sample under hybridizing conditions with the promoter-primer such that a promoter-primer extension product is synthesized; and
- (3) contacting the test sample under hybridizing conditions such that a secondary primer extension product is synthesized.

15

The present invention is directed to improved methods for determining the presence of a specific nucleic acid sequence in a test sample, and kits containing reagents necessary for practicing the invention. In general, the methods of the invention involve the synthesis of a double-stranded nucleic acid containing the nucleic acid sequence to be detected and a promoter, the synthesis of a multiplicity of RNA transcripts under the control of the promoter, and the detection of the specific RNA transcripts produced. The advantage obtained by amplification in the form of RNA transcripts rather than DNA primer extension products is that the synthesis of RNA transcripts, in the presence of an enzyme for polymerization and ribonucleoside triphosphates, occurs continuously, thus producing any desired level of amplification of the nucleic acid sequence to be detected without resort to repeated cycles of exacting and inherently error-prone primer hybridization reactions.

20

25

30

#### Brief Description of the Drawings

35

Fig. 1 illustrates the hybridization of an oligonucleotide promoter-primer to a specific sequence to be detected within a

-7-

single-stranded nucleic acid, synthesis of a double-stranded nucleic acid which comprises the sequence to be detected and the promoter of the promoter-primer, and the synthesis of a multiplicity of RNA transcripts from the double-stranded nucleic acid, under the control of the promoter.

Fig. 2 depicts an embodiment of the invention wherein an oligonucleotide promoter-primer is used in concert with an oligonucleotide secondary primer to produce a double-stranded nucleic acid containing the sequence to be detected and the promoter of the promoter-primer. The promoter-primer is hybridized to the sequence to be detected within a single-stranded nucleic acid and the secondary primer is hybridized to a sequence within the promoter-primer extension product which is complementary to the sequence to be detected. The resulting product is a double-stranded nucleic acid, wherein one strand comprises an oligonucleotide promoter-primer extension product and the other strand comprises an oligonucleotide secondary primer extension product, from which RNA transcripts are synthesized under the control of the promoter.

Fig. 3 depicts an additional embodiment of the invention wherein an oligonucleotide promoter-primer is used in concert with an oligonucleotide secondary primer. In this instance, the oligonucleotide secondary primer is hybridized to the sequence to be detected within a single-stranded nucleic acid and the oligonucleotide promoter-primer is hybridized to a sequence within the secondary primer extension product which is complementary to the sequence to be detected.

#### Detailed Description of the Invention

The term "promoter" as used herein refers to a nucleic acid sequence at which an RNA polymerase enzyme binds and initiates the synthesis of RNA transcripts. The promoter is preferably a dedicated promoter, and the RNA polymerase is preferably a

-8-

dedicated RNA polymerases. The term "dedicated" refers to the ability of the promoter to be recognized substantially only by an RNA polymerase other than that which is typically present in the test sample to be assayed. Similarly, the dedicated RNA polymerases will bind only to or preferentially to the dedicated promoter as compared to other promoters present in the test sample, and synthesize RNA transcripts of any nucleic acid sequence located downstream from the promoter. The combination of a dedicated promoter and a dedicated RNA polymerase results in an unusually specific promoter-RNA polymerase interaction.

Typically, the dedicated promoter and dedicated RNA polymerase will be obtained from the same source, as for example bacteriophage T7 or bacteriophage SP6. In the case of both T7 and SP6, the phage encoded RNA polymerase efficiently initiates synthesis of RNA transcripts only at the cognate phage promoter. RNA transcripts resulting from initiation at other prokaryotic or eucaryotic promoters are rarely observed. Moreover, the transcription reaction, consisting of a simple salt buffer, double-stranded nucleic acid template, ribonucleoside triphosphates, and phage RNA polymerase, results in rapid synthesis of large amounts of RNA. The promoter is desirably selected or its nucleotide sequence modified from the native sequence in order to minimize any adventitious hybridization to any known sequences in the test sample DNA.

The term "primer" as used herein refers to an oligonucleotide sequence, whether occurring naturally or produced synthetically, which is substantially complementary or homologous to all or part of a nucleic acid sequence to be detected. The primer must be sufficiently long to hybridize with a template nucleic acid containing the sequence to be detected or its complement, and to prime the synthesis of an extension product in the presence of an agent for polymerization. The exact length of the primer will depend on many factors, including hybridization and primer

-9-

extension synthesis reaction conditions, and the composition of the specific nucleic acid sequence to be detected. Typically the primer will contain 10-25 or more nucleotides, although it may contain fewer nucleotides. It is not necessary, however, that the primer reflect the exact sequence of the nucleic acid sequence to be detected or its complement. For example, non-complementary bases can be interspersed into the primer, or complementary bases deleted from the primer, provided that the primer is capable of hybridizing specifically with the nucleic acid sequence to be detected, or its complement, under the conditions chosen.

The term "promoter-primer" as used herein refers to an oligonucleotide whether occurring naturally or produced synthetically which comprises a promoter joined to the 5' end of a primer. Under suitable conditions and in the presence of an agent for polymerization, the promoter-primer is capable of acting as a point of initiation of a promoter-primer extension product that includes the nucleic acid sequence to be detected, or its complementary sequence, and the promoter of the promoter-primer. The promoter-primer is preferably single-stranded for maximum efficiency of hybridization, but may alternatively be double-stranded. If double-stranded, the promoter-primer is first treated to separate its strands before being used to prepare extension products.

The term "secondary primer" as used herein refers to an oligonucleotide whether occurring naturally or produced synthetically, which under suitable conditions and in the presence of an agent for polymerization, is capable of acting as a point of initiation for a secondary primer extension product that includes the nucleic acid sequence to be detected or its complement. The secondary primer is preferably single-stranded for maximum efficiency of hybridization, but may alternatively be double-stranded. If double-stranded, the secondary primer is first treated to separate its strands before being used to prepare

-10-

extension products. The secondary primer may consist of any nucleotide sequence which is substantially complementary or homologous to all or part of a nucleic acid sequence to be detected, but is preferably selected so as not to be complementary to the promoter-primer.

The term "probe" as used herein refers to an oligonucleotide whether occurring naturally or produced synthetically, which is either homologous or complementary to all or part of a nucleic acid sequence to be detected. The probe is preferably selected so that under appropriate conditions it is capable of hybridizing specifically to RNA transcripts of the nucleic acid sequence to be detected.

The term "oligonucleotide" as used herein in reference to primers and probes is defined as a nucleic acid molecule comprised of two or more deoxyribonucleotides or ribonucleotides. A desired oligonucleotide may be prepared by any suitable method, such as purification from a naturally occurring nucleic acid, or de novo synthesis. Several methods have been described in the literature, for example, for the synthesis of oligonucleotides from nucleoside derivatives using various techniques of organic chemistry. One type of organic synthesis is the phosphotriester method, wherein phosphotriester nucleosides are joined together to form an oligonucleotide with a desired sequence (Narang, et al., 1979, Meth. Enzymol. 68:90). Other methods of organic synthesis which have been described involve the use of phosphodiester nucleosides (Brown, et al., 1979, Meth. Enzymol. 68:109), or phosphoramidate nucleosides (Caruthers, et al., 1985, Meth. Enzymol. 154:287). Oligonucleotides synthesized by any of these methods may subsequently be joined together to form a single oligonucleotide of any required length and sequence. Alternatively, oligonucleotides are produced by *in vitro* transcriptional amplification, by cloning in host cells or by recovery from natural sources by the use of appropriate restriction enzymes.

-11-

The term "RNA transcript" as used herein refers to a ribonucleic acid molecule synthesized by an RNA polymerase enzyme under the control of the promoter of the promoter-primer. The RNA transcript of a specific nucleic acid sequence to be detected is either homologous or complementary to that sequence, depending upon the nature of the promoter-primer.

The term "extension product" as used herein refers to a nucleic acid molecule, the synthesis of which is initiated at the 3'-OH terminus of a primer, using as a template for synthesis the nucleic acid molecule to which the primer is hybridized.

The term "agent for polymerization" as used herein is generally understood to refer to any enzyme that catalyzes the synthesis of a nucleic acid molecule from deoxyribonucleotides or ribonucleotides, using an existing nucleic acid as a template.

Any source of nucleic acid, in purified or non-purified form, can be utilized as the test sample. For example, the test sample may be a food or agricultural product, or a human or veterinary clinical specimen. Typically, the test sample is a biological fluid such as urine, blood, plasma, serum, sputum or the like. The nucleic acid to be detected in the test sample is DNA or RNA, including messenger RNA, from any source, including bacteria, yeast, viruses, and the cells or tissues of higher organisms such as plants or animals. Methods for the extraction and/or purification of such nucleic acids have been described, for example, by Maniatis, et al., Molecular Cloning: A Laboratory Manual (New York, Cold Spring Harbor Laboratory, 1982).

The nucleic acid sequence to be detected in the test sample may be present initially as a discrete molecule so that the sequence to be detected constitutes the entire nucleic acid, or may only be a component of a larger molecule. It is not necessary that



-12-

th nucleic acid sequence to be detected be present initially in a pure form. The test sample may contain a complex mixture of nucleic acids, of which the nucleic acid sequence to be detected comprises a minor fraction. For example, the nucleic acid sequence to be detected may correspond to an oncogene contained in total human genomic DNA, or a portion of the nucleic acid sequence of a pathogenic organism which organism is a minor component of a clinical sample.

Any nucleic acid sequence may be detected by the present invention. It is only necessary that a sufficient number of nucleotides of the sequence be known so that at least one and preferably two primers may be prepared that are capable of hybridizing with the nucleic acid sequence to be detected or its complement. The nucleic acid sequence to be detected may be ascertained by any of the known methods of nucleic acid sequencing, for example, or may be predicted based upon a determined protein sequence. The greater the knowledge of the nucleic acid sequence to be detected, the greater can be the specificity of the primers selected, and thus the greater the accuracy of the invention.

Sufficient sequence information should be available, for example, to allow the selection of a promoter-primer which will hybridize specifically with the nucleic acid sequence to be detected, or its complement, but no other sequence in the test sample, under the hybridization conditions chosen. If both a promoter-primer and a secondary primer are to be used, it is desirable that the nucleic acid sequence to be detected be known to such an extent that the primers may be selected so as not to be complementary to one another.

Referring to the methods depicted in Figs. 1-3, each has as its core initial step the preparation of a double-stranded nucleic acid which includes the sequence to be detected and a promoter. In general, the preparation of this double-stranded nucleic acid

-13-

involves the synthesis of a promoter-primer extension product and optionally, a secondary primer extension product, using as a template a single-stranded nucleic acid to which the primer has hybridized. Depending upon whether a promoter-primer is used alone or in combination with a secondary primer, the template will therefore comprise a test sample nucleic acid which contains the sequence to be detected or a previously synthesized primer extension product which contains the complement of the nucleic acid sequence to be detected.

10

If the template is originally contained in a double-stranded nucleic acid, it is necessary to separate the strands of the nucleic acid before or simultaneous with the synthesis of a primer extension product. Strand separation is desirably accomplished by heat denaturation, wherein the test sample is heated to about 90°-100°C for times ranging from about 1-10 minutes, although other physical, chemical, or enzymatic denaturation procedures may be used.

15

20

Primer hybridization is typically performed in a buffered aqueous solution, for which the conditions of temperature, salts concentration, and pH are selected to provide sufficient stringency such that the primer will hybridize specifically to the nucleic acid sequence to be detected, or its complement, but not any other sequence. Generally, the efficiency of hybridization between primer and template will be improved under conditions where the amount of primer added is in molar excess to the template, preferably a 1000 to  $10^6$  molar excess. It is understood, however, that the amount of template in the test sample may not be known, so that the amount of primer relative to template cannot be determined with certainty.

25

30

Fig. 1A-G depict one embodiment of the present invention wherein a promoter-primer is used to prepare a double-stranded

-14-

nucleic acid which includes the sequence to be detected and a promoter.

5 In Fig. 1A, the primer c of the promoter-primer c d is shown hybridizing with the nucleic acid sequence to be detected b as part of a nucleic acid a, through non-covalent base pairing e of complementary nucleotide sequences. The promoter d of the promoter-primer, being preferably selected so as not to be complementary with any known nucleic acid sequence in the test sample, remains unhybridized.

10 The subsequent addition of a polynucleotide polymerase f, in the presence of nucleoside triphosphates, results (Fig. 1B-C) in the synthesis of a promoter-primer extension product g, initiating at the 3' end of the primer and proceeding in the 5' direction along the single-stranded template, which template consists of the test sample nucleic acid sequence a flanking on the 5' side of the sequence b within which the primer is originally hybridized. The primer extension product is thus complementary to the nucleic acid sequence to be detected and the 5'-flanking test sample nucleic acid sequence, if any, forming a double-stranded molecule.

20 In the presence of an agent possessing 3'-5' exonuclease activity h, the single-stranded sequence at the 3' end of the test sample nucleic acid, if any, is excised as shown in Fig. 1D, and replaced by new nucleic acid (Fig. 1E-G), the synthesis of which is initiated by a polynucleotide polymerase f at the 3' end of the test sample nucleic acid using as a template the promoter of the promoter-primer.

30

The method depicted in Fig. 1A-G for generating a double-stranded molecule which includes the sequence to be detected and a promoter depends upon hybridization of a promoter-primer to the nucleic acid sequence to be detected and subsequent steps of promoter-primer extension synthesis, nucleic acid excision, and

35

-15-

replacement synthesis. Although the excision reaction may be carried out by an agent h different from that used for promoter-primer extension synthesis and replacement synthesis f, it is desirable to use a single agent such that the synthesis of the double-stranded product may be carried out in a continuous manner following promoter-primer hybridization. Accordingly, the polynucleotide polymerase is preferably one which possesses both 5'-3' polymerase activity and 3'-5' exonuclease activity, such as, for example, E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, and T4 DNA polymerase. As each of these enzymes requires a DNA template for primer extension synthesis, it is understood that their use according to the method depicted in Fig. 1B and 1F will be appropriate in instances where the nucleic acid sequence to be detected is DNA.

The double-stranded nucleic acid which includes the sequence to be detected and a promoter serves as the source of a multiplicity of RNA transcripts of the sequence to be detected. As depicted in Fig. 1H-I, addition of a suitable RNA polymerase aa to the test sample results in the binding of the RNA polymerase to the promoter of the double-stranded product, and in the presence of ribonucleoside triphosphates, the subsequent synthesis of RNA transcripts bb of the nucleic acid sequence located downstream from the promoter, including the nucleic acid sequence to be detected.

Under suitable reaction conditions, including the presence of the necessary reagents, the synthesis of RNA transcripts will occur continuously and in proportion to the amount of the nucleic acid sequence to be detected that was originally present in the test sample. Additional reagents may be added as necessary to prepare the desired quantity of RNA transcripts. Preferably the synthesis of RNA transcripts will be carried out in the presence of a ribonuclease inhibitor, as for example vanadyl-ribonucleoside complexes or human placental ribonuclease inhibitor, in order to avoid possible degradation of the transcripts by any adventitious

-16-

ribonuclease contaminant. Berger, 1987, Meth. Enzymol. 152:227; de Martynoff et al., 1980, Bi chem. Biophys. Res. Commun. 93:645; Sheel et al., 1979, Proc. Natl. Acad. Sci. 76:4898. After the appropriate length of time has passed to produce the desired quantity of RNA transcripts, the reaction may be halted by inactivating the RNA polymerase in any known manner or separating the components of the reaction.

Determination of the specific RNA transcripts produced is accomplished by any suitable method, as for example, by labeling of the RNA transcripts with a detectable moiety during or after their synthesis, or by the use of a labeled probe capable of hybridizing specifically with RNA transcripts of the nucleic acid sequence to be detected.

RNA transcripts may be labeled, for example, by providing ribonucleoside triphosphates which are themselves labeled with a detectable moiety, and which are utilized as a substrate by RNA polymerase and thereby incorporated into RNA transcripts. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. In one embodiment, for example, the detectable moiety may be a radioisotope, such as  $^{32}\text{P}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ , and  $^{35}\text{S}$ . In a further embodiment, the detectable moiety may be a non-radioactive chemical modification capable of producing a detectable signal upon interaction with one or more appropriate agents. Thus, for example, the detectable moiety can be biotin, and the detectable signal produced by the formation of a complex between the biotin moiety and a protein capable of binding specifically to biotin, as for example avidin, streptavidin, or anti-biotin antibody, which binding protein is conjugated with a detectable molecule, like fluorescein, or with an enzyme which reacts with a suitable substrate to form a fluorescent, luminescent, or colored product. Langer et al., 1981, Proc. Natl. Acad. Sci. 78:6633; Bayer and Wilchek, 1980, Meth. Biochem. Anal. 26:1.

-17-

In an additional embodiment, the specific RNA transcript produced may be determined by the use of a nucleic acid hybridization probe. Falkow et al., U.S. Pat. No. 4,358,535; Goodson et al., European Pat. App. 0 238 332. The probe is desirably selected so as to hybridize within the sequence of the RNA transcript which is either complementary or homologous to the nucleic acid sequence to be detected. The hybridization method may be carried out by any suitable method, including the liquid hybridization methods described in European Pat. Publication Nos. 70,685 and 70,687, and the liquid-solid hybridization methods described in U.S. Pat. Nos. 4,358,535 (Falkow) and 4,647,529 (Rodland), European Pat. App. 0 238 332 (Goodson), and Ranki, et al., 1983, Gene 21:77. In the case of liquid-solid hybridization, either the probe molecules or the RNA transcripts can be immobilized on the solid support.

Any suitable method may be used for correlating the amount of RNA transcripts with the amount of nucleic acid sequence to be detected in the test sample, including, for example, determination of a fixed quantity of a standard nucleic acid which standard nucleic acid contains the sequence to be detected or another known sequence, simultaneous or in parallel with determination of the sequence to be detected in the test sample. Ranki, et al., UK Pat. App. 2 187 283 A.

In further embodiments of the invention, the synthesis of a double-stranded nucleic acid containing the sequence to be detected and a promoter is accomplished using a promoter-primer in concert with a secondary primer to initiate the synthesis of two different primer extension products that are capable of hybridizing to one another.

Fig. 2A-F illustrate the method wherein the synthesis of the promoter-primer extension product is carried out first, and is

-18-

followed by the synthesis of the secondary primer extension product. In Fig. 2A, the primer c of the promoter-primer c d is shown hybridizing at the 3' end of the nucleotide sequence to be detected b, through non-covalent base pairing a of complementary nucleotide sequences. Upon addition of an agent for polymerization f, and in the presence of nucleoside triphosphates, an extension product g is synthesized initiating at the 3' end of the promoter-primer and proceeding in the 5' direction along the single-stranded template. The resulting product is thus a double-stranded nucleic acid, of which one strand is the test sample nucleic acid and the other is the promoter-primer extension product g.

In the next step (Fig. 2D), the two strands are separated using any of the procedures described above to provide single-stranded molecules. Following this strand separation step, the test sample is contacted with the secondary primer h under conditions suitable for hybridization of the secondary primer to the sequence within the promoter-primer extension product which is complementary to the sequence to be detected. As it is desirable, however, that the secondary primer not be complementary to the promoter-primer in order to improve the accuracy of the assay, the secondary primer is preferably selected to hybridize at the 3' end of the complementary sequence (Fig. 2E).

In the continued presence of the agent for polymerization used for promoter-primer extension synthesis f, or upon addition of an agent for polymerization i, a secondary primer extension product is synthesized, initiating at the 3' end of the secondary primer and proceeding in a 5' direction along the promoter-primer extension product template (Fig. 2F). The secondary primer extension product will thus be complementary to the promoter-primer extension product and hybridize therewith to form a double-stranded nucleic acid which includes the sequence to be detected and the promoter of the promoter-primer.

35

-19-

The agent for polymerization which is used to synthesize the promoter-primer extension product is preferably exonuclease deficient T7 DNA polymerase (Taber and Richardson, 1987, Proc. Natl. Acad. Sci. 84:4767), although other agents may be used, including reverse transcriptase and other polynucleotide polymerases deficient in 3'-5' exonuclease activity. To the extent that commercially available exonuclease deficient T7 DNA polymerase (Sequenase<sup>TM</sup>, United States Biochemical Corp., Cleveland, Ohio) possesses residual 3'-5' exonuclease activity, its usage may be undesirable in certain instances, as for example, where the unhybridized 3' end of the test sample deoxyribonucleic acid to which a promoter-primer is bound may be excised before the strand separation step shown in Fig. 2D. This may be expected to occur in circumstances where the test sample nucleic acid is mechanically sheared or digested with a restriction enzyme, thereby reducing the length of the test sample nucleic acid sequence on the 3' side of the sequence to which the promoter-primer is bound.

Synthesis of the secondary primer extension product is accomplished using any agent for polymerization which is capable of initiating synthesis at the 3' end of the secondary primer and proceeding in the 5' direction along the promoter-primer extension product template. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, exonuclease deficient T7 DNA polymerase, and reverse transcriptase.

Fig. 3A-I illustrate the method wherein the synthesis of a secondary primer extension product is carried out first and is followed by the synthesis of a promoter-primer extension product. In Fig. 3A the secondary primer *c* is shown hybridizing at the 3' end of the nucleotide sequence to be detected *b*, through non-covalent base pairing *d* of complementary nucleotide sequences. Upon addition of an agent for polymerization *g*, and in the presence of nucleoside triphosphates, an extension product *f* is synthesized



-20-

initiating at the 3' end of the secondary primer and proceeding in the 5' direction along the single-stranded template. The resulting product (Fig. 3C) is thus a double-stranded nucleic acid, of which one strand is the test sample nucleic acid and the other is the secondary primer extension product.

In the next step (Fig. 3D), the two strands are separated using any of the procedures described above to provide single-stranded molecules. Following this strand separation step, the test sample is contacted with the promoter-primer *g* under conditions suitable for hybridization of the promoter-primer to the sequence within the secondary primer extension product which is complementary to the nucleic acid sequence to be detected. As it is desirable that the promoter-primer not be complementary to the secondary primer in order to improve the accuracy of the invention, the primer of the promoter-primer is preferably selected to hybridize at the 3' end of the complementary sequence.

In the continued presence of the agent for polymerization used for secondary primer extension synthesis *a*, or upon addition of an agent for polymerization *i*, a promoter-primer extension product *j* is synthesized, initiating at the 3' end of the promoter-primer and proceeding in a 5' direction along the secondary primer extension product template. The promoter-primer extension product includes the nucleic acid sequence to be detected, which sequence is hybridized to its complement within the secondary primer extension product, and the promoter. The resulting product is thus a double-stranded nucleic acid which includes the sequence to be detected and the promoter of the promoter-primer.

In the presence of an agent *k* possessing 3',-5' exonuclease activity, the single-stranded sequence at the 3' end of the promoter-primer extension product, if any, is excised and replaced by a promoter complementary sequence (Fig. 3G-I), the synthesis of which is initiated by an agent for polymerization *i* at the 3' end

-21-

f th promoter-primer extension pr duct using as a template the prom ter of the promoter-primer.

5 Synthesis of the secondary primer extension product is accomplished using any agent for polymerization which is capable f initiating synthesis at the 3' end of the secondary primer and proceeding in the 5' direction along the test sample nucleic acid template. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, exonuclease deficient T7 DNA polymerase, and reverse transcriptase. Synthesis of the promoter-primer extension product and synthesis of the promoter complementary sequence may be accomplished using the same agent or different agents. including the agent for polymerization used for secondary primer extension synthesis. To improve the efficiency of the process depicted in Fig. 3, it is desirable, however, that the agent used for synthesis of the promoter-primer extension product also be capable of carrying out the excision reaction shown in Fig. 3G, whereby any unhybridized sequence at the 3' end of the secondary primer extension product is removed. Enzymes suitable for carrying ut both the synthesis and excision reactions include th se polynucleotide polymerases that possess 3'-5' exonuclease activity, such as *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, and T4 DNA polymerase.

25

Once formed, the double-stranded nucleic acid product of this or the preceding method serves as the source of a multiplicity of RNA transcripts of the sequence to be detected, the synthesis and determination of which may be accomplished using any of the procedures described above.

30

One advantage obtained from the use of a promoter-primer and a secondary primer in combination to produce a double-stranded nucleic acid which includes the sequence to be detected and a promoter is greater accuracy of the assay. In the event that

35

-22-

either the promoter-primer or secondary primer erroneously hybridizes to a sequence other than the sequence to be detected or its complement, the resulting primer extension side-product is not expected, whether by hybridization to the test sample nucleic acid or to another primer extension product, to produce a double-stranded product from which RNA transcripts will be synthesized.

Another advantage of using the two primers in combination is that the RNA transcripts of the sequence to be detected will be of a discrete size. As depicted in Figs. 2 and 3, by selecting the promoter-primer and the secondary primer such that one primer hybridizes at the 3' end of the sequence to be detected, and the other primer hybridizes at the 3' end of the complementary sequence, the double-stranded region of the nucleic acid product from which RNA transcripts are synthesized may be limited to the sequence to be detected and the promoter of the promoter-primer. The discrete size of the resulting RNA transcripts may be advantageous to the subsequent detection or isolation of the transcripts, as for example, separation of the desired RNA transcripts from the test sample by gel filtration chromatography. Furthermore, to the extent that the transcripts produced are shorter than those produced according to the method depicted in Fig. 1, their synthesis will proceed more rapidly and require lesser amounts of reagents.

25

The following examples are offered by way of illustration, and are not intended to limit the invention in any manner. All references described herein are expressly incorporated.

30

#### Example 1

This example illustrates the use of a deoxyribonucleotide promoter-primer having the sequence

5'AAATTAATACGACTCACTATAGGGAGATGTACCTCTGTATCATATGC 3'

for the detection of the env gene of HIV (human immunodeficiency virus; formerly called HTLV-III/LAV). The sequence of this

35

-23-

promoter-primer is selected such that the 5' end of the promoter-primer corresponds to the sequence of the functional domain of a bacteriophage T7 class III promoter (Dunn and Studier, 1983, J. Mol. Biol. 166:477), and the 3' end is complementary to a portion of the coding sequence of the env gene of HIV, between nucleotides 5981-6000 of the HIV genomic sequence (Muessing et al., 1985, Nature 313:450).

Nucleic acid for analysis is extracted from test samples of citrate-treated human blood or virus-infected H9 cells (Popovic, et al., 1984, Science 224:497) using the techniques described by Hermann and Fischau, 1987, Meth. Enzymol. 152:180.

A total of 10 µg of test sample nucleic acid is added to 100 pmoles of promoter-primer in 100 µl of reaction buffer containing 40mM Tris HCl (pH 8.0), 20mM MgCl<sub>2</sub>, 1mM dithiothreitol, 5 mg/ml gelatin, 10mM vanadyl-ribonucleoside complexes, and 10mM each of the four deoxyribonucleoside triphosphates (dATP, dTTP, dGTP, dCTP) and the four ribonucleoside triphosphates (ATP, UTP, GTP, CTP). The mixture is heated at 100°C for one minute, and allowed to cool to 37°C. 5 units of Klenow fragment of *E. coli* DNA polymerase I and 5 units of T7 RNA polymerase are then added to the mixture and the reactions allowed to proceed for one hour at 37°C.

The RNA transcripts produced are detected by dot-blot hybridization, using as a hybridization probe a <sup>32</sup>P end-labeled deoxyoligonucleotide having the sequence

5' TTGATGATCTGTAGTGCTAC 3'.

The sequence of this probe was selected to be homologous to a portion of the coding sequence of the HIV env gene, located between nucleotides 5875 and 5895 of the HIV genomic sequence.

Aliquots from the RNA synthesis reaction are added to 100 µl of 15X SSC buffer (1X SSC = 0.15M sodium chloride, 0.015M sodium citrate, pH 7.0) and filtered through nitrocellulose filters prewet

-24-

in 6X SSC. The filters are then baked for one hour at 80°C in a vacuum oven.

5 After baking, each filter is contacted with hybridization solution consisting of 6X SSC, 50mM sodium phosphate (pH 6.5), 5X Denhardt's solution (1X = 0.02% polyvinylpyrrolidone, 0.02% Fic 11, 0.02% bovine serum albumin, 0.2mM Tris HCl, 0.2mM EDTA, pH 8.0), 0.1% sodium dodecyl sulfate, and 0.2% denatured salmon sperm DNA  
10 for one hour at 42°C. The labeled probe is then added to the hybridization solution to a final concentration of  $10^7$  cpm/ml and incubation of the filters continued for two hours at 42°C.

15 Finally, the filters are washed in 6X SSC for one hour at 37°C with two changes of buffer. After drying, the filters are autoradiographed or counted for Cerenkov radioactivity.

#### Example 2

20 This and the following example illustrate the use of a promoter-primer in combination with a secondary primer for the detection of the env gene of HIV.

10 µg of test sample nucleic acid prepared from human blood or virus-infected H9 cells as described in Example 1 is mixed with 100 pmoles of promoter-primer and 100 pmoles of secondary primer (5' ATGAGAGTGAAGGAGAAATA 3') in 100 µl of reaction buffer. This  
25 secondary primer is homologous to the amino-terminal coding sequence of the env gene of HIV (nucleotides 5803-5822). This specific combination of promoter-primer and secondary primer was selected such that a 225 base-pair double-stranded nucleic acid  
30 will be produced, containing 198 base-pairs of the sequence of the env gene of HIV, from which a 206 nucleotide RNA transcript will be synthesized under the transcriptional control of a flanking T7 promoter.

-25-

The mixture of the two primers and the test sample nucleic acid is heated at 100°C for one minute and allowed to cool to 37°C whereupon 5 units of exonuclease deficient T7 DNA polymerase (Sequenase<sup>TM</sup>, United States Biochemical Corp.) is added. After incubation at 37°C for 5 minutes, the reaction mixture is again heated at 100°C for one minute and allowed to cool to 37°C. 5 units of Sequenase<sup>TM</sup> and 5 units of T7 RNA polymerase are then added to the mixture, and the reaction allowed to proceed at 37°C for 30 minutes to one hour. Finally, the reaction mixture is heated at 100°C for 5 minutes and allowed to cool to 42°C.

The resulting RNA transcripts are determined by reverse transcription which is specifically primed by the hybridization of the secondary primer with the nascent RNA transcripts.  $\alpha$ -<sup>32</sup>P-dTTP (specific activity approx. 3000 Ci/mmol) is added to the test sample reaction mixture to a final concentration of 1  $\mu$ M, together with 5 units of reverse transcriptase, and the mixture is then incubated for 15 minutes at 42°C.

<sup>32</sup>P-labeled reverse transcription products are quantitated by determining the total acid insoluble radioactivity in the reaction mixture. Aliquots of the reaction mixture are added to 500  $\mu$ l of 10% trichloroacetic acid (TCA) contained in polystyrene tubes and incubated on ice for 10 minutes. The contents of each tube is then filtered through a Whatman glass fiber filter with suction and the filter washed with ice cold TCA. After drying, the filters are counted for Cerenkov radioactivity.

### Example 3

Nucleic acid isolated from human blood or HIV-infected H9 cells is sheared by sonication to produce fragments with an average length of 500 nucleotides. 10  $\mu$ g of sheared test sample nucleic acid is then mixed with 100 pmoles of promoter-primer and 100 pmoles of secondary primer in 100  $\mu$ l of buffer containing 40mM Tris HCl (pH 8.0), 20mM MgCl<sub>2</sub>, 1mM dithiothreitol, 5 mg/ml gelatin, 10mM

-26-

vanadyl-ribonucleoside complexes, 10 mM each of the four deoxyribonucleoside triphosphates (dATP, dTTP, dGTP, dCTP), 500  $\mu$ M ATP, 500  $\mu$ M UTP, 500  $\mu$ M GTP, 500  $\mu$ M CTP, and 1  $\mu$ M  $\alpha$ -<sup>32</sup>P-UTP (specific activity approx. 3000 Ci/mmol). The mixture is heated at 100°C for one minute and allowed to cool to 28°C, whereupon 5 units of reverse transcriptase is added. After incubation at 28°C for 15 minutes, the reaction mixture is reheated at 100°C for one minute, and allowed to cool to 28°C. 5 units of reverse transcriptase and 5 units of T7 DNA polymerase are then added, and the reactions allowed to proceed for one hour at 28°C.

<sup>32</sup>P-labeled RNA transcripts are quantitated by determining the total acid insoluble radioactivity in the reaction mixture, as described above. The 206 nucleotide labeled RNA transcript may also be determined by autoradiography of urea-polyacrylamide gels on which aliquots of the RNA synthesis reaction are run in parallel with various amounts of radiolabeled standard RNAs. Quantitation of the 206 nucleotide transcript of the gny gene sequence is then accomplished by densitometry analysis of the developed autoradiogram.

-27-

Claims

1. A method for the detection of a specific nucleic acid sequence in a test sample containing a nucleic acid which comprises:
  - 5 (a) synthesizing a double-stranded nucleic acid which includes the sequence to be detected and a promoter;
  - (b) synthesizing a multiplicity of RNA transcripts from said double-stranded nucleic acid under the control of said promoter;  
10
  - (c) determining the presence of RNA transcripts produced in step (b); and
  - 15 (d) correlating the presence of said RNA transcripts with the presence of the nucleic acid sequence to be detected.
2. The method of claim 1 wherein the specific nucleic acid sequence to be detected in the test sample is contained in double-stranded DNA, and the two strands are separated by denaturing before or during step (a).  
20
3. The method of claim 1 wherein the specific nucleic acid sequence to be detected in the test sample is contained in RNA or single-stranded DNA.  
25
4. The method of claim 1 wherein the specific nucleic acid sequence to be detected is characteristic of a genetic disease, or is homologous to a sequence contained in a pathogenic organism or in an oncogene.  
30
5. The method of claim 4 wherein the pathogenic organism is a retrovirus or a bacterium.



-28-

6. The method of claim 4 wherein the pathogenic organism is a human immunodeficiency virus.
- 5 7. The method of claim 1 wherein the promoter is a bacteriophage T7 promoter, and the multiplicity of RNA transcripts is synthesized using T7 RNA polymerase.
8. The method of claim 1 wherein the promoter is a bacteriophage SP6 promoter, and the multiplicity of RNA transcripts is synthesized using SP6 RNA polymerase.  
10
9. The method of claim 1 wherein the test sample nucleic acid is digested with a restriction enzyme before or during step (a).
- 15 10. The method of claim 1 wherein the double-stranded nucleic acid of step (a) is obtained by the steps comprising:
  - (1) providing an oligonucleotide promoter-primer comprising a promoter ligated to a primer;  
20
  - (2) contacting the test sample with the promoter-primer under conditions that permit the hybridization of the promoter-primer to the nucleic acid sequence to be detected; and
  - 25 (3) synthesizing an extension product from the promoter-primer which is complementary to the nucleic acid sequence to be detected, using as a template the test sample nucleic acid to which the promoter-primer has hybridized in step (2).  
30
11. The method of claim 10 wherein the synthesis of the promoter-primer extension product is accomplished using an enzyme selected from the group consisting of *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, and T4 DNA  
35 polymerase.

-29-

12. The method of claim 1 wherein the double-stranded nucleic acid of step (a) is obtained by the steps comprising:

5 (1) providing an oligonucleotide promoter-primer, comprising a promoter ligated to a primer, and an oligonucleotide secondary primer, wherein said primers are not complementary to one another, and are selected such that the extension product synthesized from one primer, when  
10 separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

15 (2) contacting the test sample under hybridizing conditions with the promoter-primer such that a promoter-primer extension product is synthesized; and

20 (3) contacting the test sample under hybridizing conditions with the secondary primer such that a secondary primer extension product is synthesized.

25 13. The method of claim 12 wherein the synthesis of the primer extension products is accomplished using exonuclease deficient T7 DNA polymerase or reverse transcriptase.

30 14. The method of claim 12 wherein the synthesis of the primer extension products is accomplished using exonuclease deficient T7 DNA polymerase and one or more enzymes selected from the group consisting of *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, and T4 DNA polymerase.

15. The method of claim 12 wherein the synthesis of the primer extension products is accomplished using reverse transcriptase and one or more enzymes selected from the group consisting of

-30-

E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, and T4 DNA polymerase.

- 5 16. The method of claim 12 wherein synthesis of the promoter-primer extension product is carried out before synthesis of the secondary primer extension product, and synthesis of the promoter-primer extension product is accomplished using exonuclease deficient T7 DNA polymerase or reverse transcriptase.
- 10 17. The method of claim 12 wherein synthesis of the secondary primer extension product is carried out before synthesis of the promoter-primer extension product, and synthesis of the secondary primer extension product is accomplished using an
- 15 enzyme selected from the group consisting of E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, and T4 DNA polymerase.
- 20 18. The method of claim 1 wherein the RNA transcripts synthesized in step (b) are labeled.
- 25 19. The method of claim 18 wherein the RNA transcripts are labeled with one or more radioisotopes and step (c) is accomplished by measuring the amount of radioactivity incorporated.
20. The method of claim 18 wherein the RNA transcripts are labeled with a detectable non-radioactive chemical modification.
- 30 21. The method of claim 1 wherein, after step (b) and before step (c), the RNA transcripts are contacted under hybridizing conditions with an oligonucleotide probe selected to hybridize with a predetermined sequence within the RNA transcripts.
- 35 22. The method of claim 21 wherein the RNA transcripts are immobilized on a solid support.

-31-

23. The method of claim 21 wherein a labeled extension product is synthesized from the oligonucleotide probe, using as a template the RNA transcripts to which the probe has hybridized, and step (c) is accomplished by measuring the amount of probe extension product.
24. The method of claim 23 wherein the probe extension product is labeled with one or more radioisotopes and step (c) is accomplished by measuring radioactivity.
25. The method of claim 23 wherein the probe extension product is labeled with a detectable non-radioactive chemical modification.
26. The method of claim 21 wherein the probe is labeled and step (c) is accomplished by measuring the amount of probe hybridized to RNA transcripts.
27. The method of claim 26 wherein the probe is labeled with one or more radioisotopes and step (c) is accomplished by measuring radioactivity.
28. The method of claim 26 wherein the probe is labeled with a detectable non-radioactive chemical modification.
29. The method of claims 20, 25, or 28 wherein the label is detected by optical analysis.
30. The method of claims 20, 25, or 28 wherein the label is biotin.
31. A method for the detection of a specific nucleic acid sequence in a test sample containing single-stranded DNA which comprises:

-32-

- 5 (a) contacting the test sample with an oligonucleotide promoter-primer, comprising a promoter for a dedicated RNA polymerase ligated to a primer, under conditions that permit hybridization of the promoter-primer to the nucleic acid sequence to be detected;
- 10 (b) contacting the test sample with a DNA polymerase, such that a double-stranded nucleic acid is synthesized, comprising the nucleic acid sequence to be detected and the promoter of the promoter-primer;
- 15 (c) contacting the product of step (b) with a dedicated RNA polymerase capable of recognizing the promoter of the oligonucleotide promoter-primer, whereby a multiplicity of RNA transcripts of the nucleic acid sequence to be detected are synthesized by the RNA polymerase under the control of said promoter;
- 20 (d) determining the presence of RNA transcripts synthesized in step (c); and
- 25 (e) correlating the presence of the RNA transcripts with the presence of the nucleic acid sequence to be detected.
32. A method for the detection of a specific nucleic acid sequence in a test sample containing RNA or single-stranded DNA which comprises:
- 30 (a) contacting the test sample with an oligonucleotide promoter-primer, comprising a promoter for a dedicated RNA polymerase ligated to a primer, under conditions that permit hybridization of the promoter-primer to the specific nucleic acid sequence to be detected;
- 35

-33-

- 5 (b) contacting the test sample with reverse transcriptase such that a promoter-primer DNA extension product is synthesized, wherein the template for synthesis of the promoter-primer DNA extension product is the test sample RNA or single-stranded DNA to which the promoter-primer has hybridized in step (a);
- 10 (c) treating the product of step (b) under denaturing conditions to separate the promoter-primer DNA extension product from its template;
- 15 (d) contacting the single-stranded nucleic acid produced in step (c) with an oligonucleotide secondary primer, wherein said secondary primer is selected to be homologous to all or part of the nucleic acid sequence to be detected and not complementary to the promoter-primer used in step (a), under conditions that permit hybridization of the secondary primer to the promoter-primer DNA extension product;
- 20 (e) contacting the product of step (d) with a DNA polymerase or reverse transcriptase, such that a secondary primer DNA extension product is synthesized, wherein the template for synthesis of the secondary promoter DNA extension product is the promoter-primer DNA extension product;
- 25 (f) contacting the product of step (e) with a dedicated RNA polymerase capable of recognizing the promoter of the oligonucleotide promoter-primer, whereby a multiplicity of RNA transcripts of the nucleic acid sequence to be detected are synthesized under the control of said promoter; and
- 30

-34-

- (g) correlating the presence of the RNA transcripts with the presence of the nucleic acid sequence to be detected.

5 33. A method for the detection of a specific nucleic acid sequence in a test sample containing RNA or single-stranded DNA which comprises:

10 (a) contacting the test sample with an oligonucleotide secondary primer, under conditions that permit hybridization of the secondary primer to the specific nucleic acid sequence to be detected;

15 (b) contacting the test sample with reverse transcriptase such that a secondary primer DNA extension product is synthesized, wherein the template for synthesis of the secondary primer DNA extension product is the test sample RNA or single-stranded DNA to which the secondary primer has hybridized in step (a);

20 (c) treating the product of step (b) under denaturing conditions to separate the secondary primer DNA extension product from its template;

25 (d) contacting the single-stranded nucleic acid produced in step (c) with an oligonucleotide promoter-primer, comprising a promoter for a dedicated RNA polymerase ligated to a primer, wherein said promoter-primer is selected to be homologous to all or part of the nucleic acid sequence to be detected and not complementary to the  
30 secondary primer used in step (a), under conditions that permit hybridization of the promoter-primer to the secondary primer DNA extension product;

35 (e) contacting the product of step (d) with a DNA polymerase such that a promoter-primer DNA extension product is

-35-

synthesized, wherein the template for synthesis of the promoter-primer DNA extension product is the secondary primer DNA extension product;

5 (f) contacting the product of step (e) with a dedicated RNA polymerase capable of recognizing the promoter of the oligonucleotide promoter-primer, whereby a multiplicity of RNA transcripts of the nucleic acid sequence to be detected are synthesized under the control of said promoter; and

10

(g) correlating the presence of said RNA transcripts with the presence of the nucleic acid sequence to be detected.

15 34. A method for the detection of a specific nucleic acid sequence in a test sample containing single-stranded DNA which comprises:

20

(a) contacting the test sample with an oligonucleotide secondary primer, under conditions that permit hybridization of the secondary primer to the specific nucleic acid sequence to be detected;

25

(b) contacting the test sample with a DNA polymerase such that a secondary primer DNA extension product is synthesized, wherein the template for synthesis of the secondary primer DNA extension product is the test sample RNA or single-stranded DNA to which the secondary primer has hybridized in step (a);

30

(c) treating the product of step (b) under denaturing conditions to separate the secondary primer DNA extension product from its template;



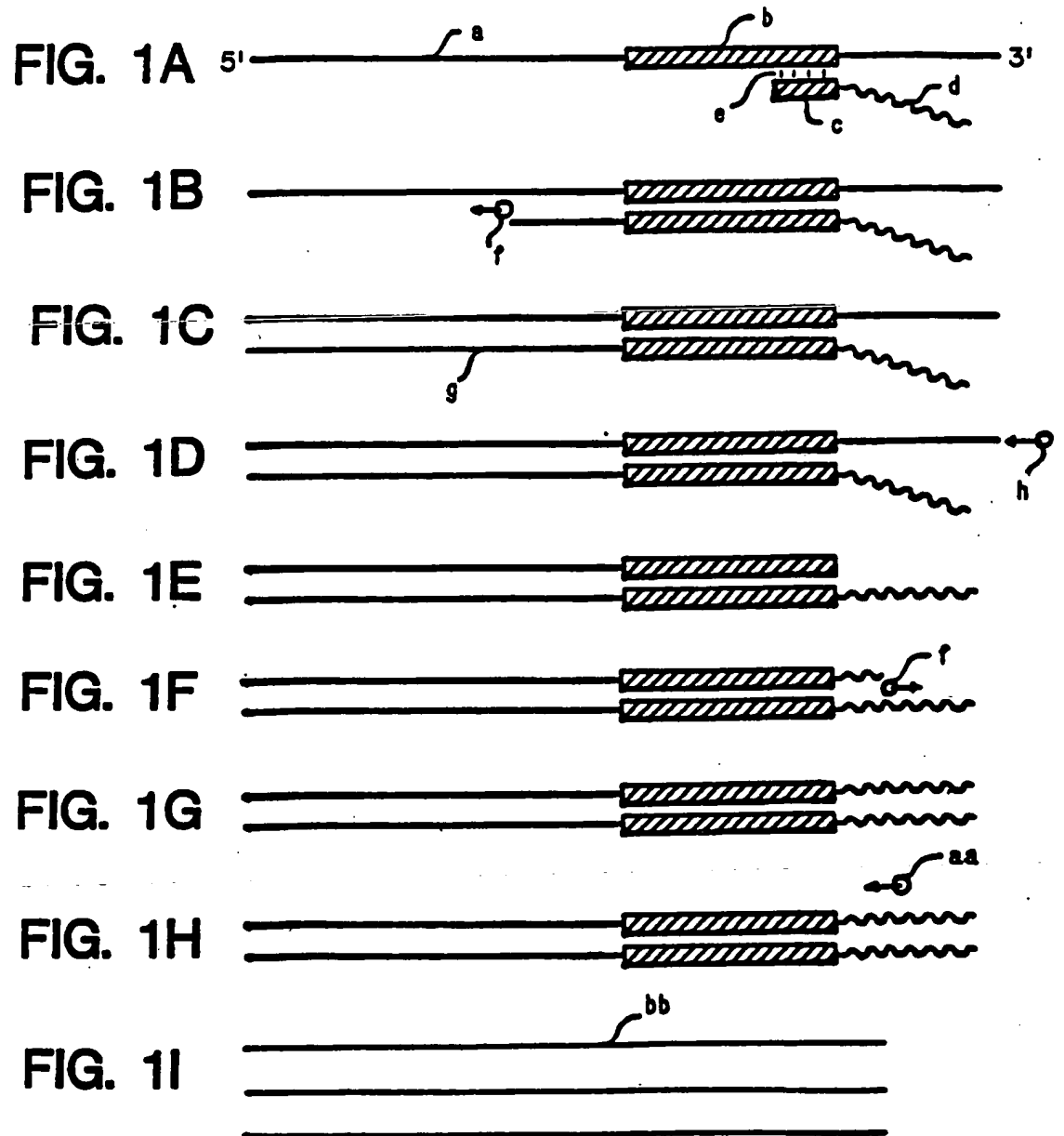
-36-

- 5 (d) contacting the single-stranded nucleic acid produced in step (c) with an oligonucleotide promoter-primer, comprising a promoter for a dedicated RNA polymerase ligated to a primer, wherein said promoter-primer is selected to be homologous to all or part of the nucleic acid sequence to be detected and not complementary to the secondary primer used in step (a), under conditions that permit hybridization of the promoter-primer to the secondary primer DNA extension product;
- 10 (e) contacting the product of step (d) with a DNA polymerase such that a promoter-primer DNA extension product is synthesized, wherein the template for synthesis of the promoter-primer DNA extension product is the secondary primer DNA extension product;
- 15 (f) contacting the product of step (e) with a dedicated RNA polymerase capable of recognizing the promoter of the oligonucleotide promoter-primer, whereby a multiplicity of RNA transcripts of the nucleic acid sequence to be detected are synthesized under the control of said promoter; and
- 20 (g) correlating the presence of said RNA transcripts with the presence of the nucleic acid sequence to be detected.
- 25
- 30 35. A kit for use in the detection of a nucleic acid sequence in a test sample containing an oligonucleotide promoter-primer and an oligonucleotide probe, wherein the promoter-primer is capable of hybridizing to the test sample nucleic acid sequence and the oligonucleotide probe is homologous to all or part of the nucleic acid sequence to be detected.

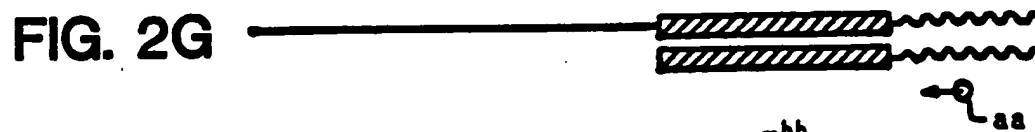
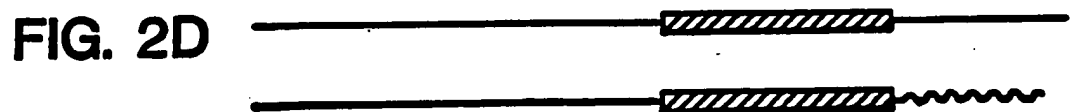
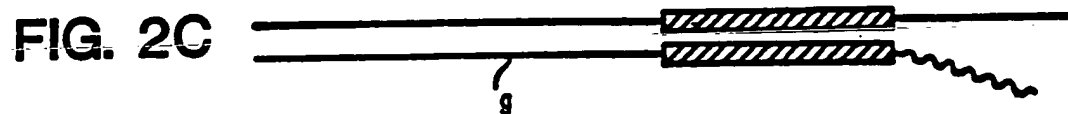
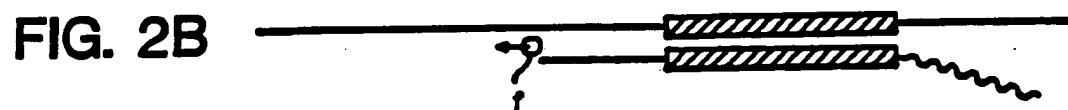
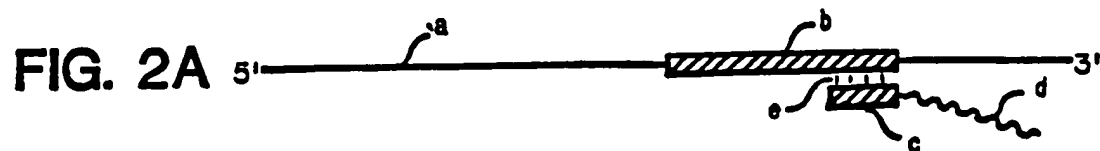
-37-

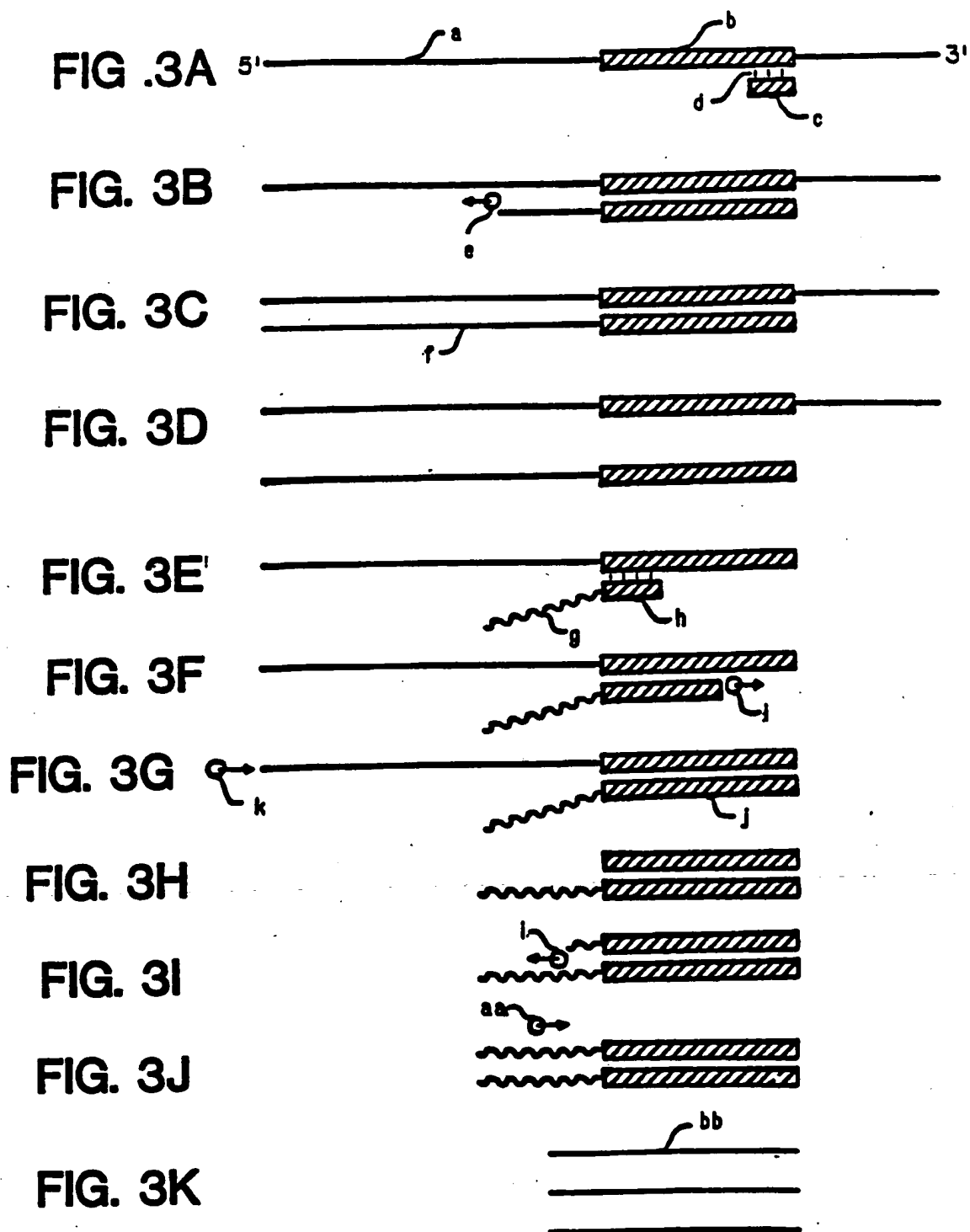
36. A kit for use in the detection of a nucleic acid sequence in a test sample containing an oligonucleotide primer and an oligonucleotide secondary primer, wherein the primers are not complementary to one another, and are selected such that one primer is capable of hybridizing to the test sample nucleic acid and the other is capable of hybridizing to the complement of the nucleic acid sequence to be detected.

5



2 / 3





# INTERNATIONAL SEARCH REP RT

International Application No

PCT/US 89/00120

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC <b>IPC4: C 12 Q 1/68</b>						
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched *</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px; vertical-align: top;">IPC4</td> <td style="padding: 5px; vertical-align: top;">C 12 Q; C 12 N; C 12 P</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *</div>			Classification System	Classification Symbols	IPC4	C 12 Q; C 12 N; C 12 P
Classification System	Classification Symbols					
IPC4	C 12 Q; C 12 N; C 12 P					
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>						
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **				
E,X	WO, A1, 88/10315 (SISKA DIAGNOSTICS, INC.) 29 December 1988, See the whole document but especially the claims.	1-36				
P,X	Scientia, Vol. 239, 1988 E.S. Stoflet et al: "Genomic Amplification with Transcript Sequencing", see page 491 - page 494 see especially figures 1 and 2 page 492.	1-4,7, 10-11, 31,35				
A	Nucleic Acids Research, Vol. 15, No. 21, 1987 John F. Milligan et al: "Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates", see page 8783 - page 8798	1-36				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>						
<b>IV. CERTIFICATION</b>						
Date of the Actual Completion of the International Search <b>10th May 1989</b>		Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold; font-size: 1.2em;">30.05.89</div>				
International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>		Signature of Authorized Officer <div style="text-align: center;"> <b>M. VAN MCL</b> </div>				

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Nucleic Acids Research, Vol. 14, No. 8, 1986 Jeanne E. Brown et al: "Sequences of three promoters for the bacteriophage SP6 RNA polymerase. ", see page 3521 - page 3526 ---	1-36
T	Bio/technology, Vol. 6, 1988 Paul M. Lizardi et al: "Exponential amplification of recombinant-RNA hybridization probes ", see page 1197 - page 1202 ---	1-36
A	EP, A2, 0236069 (CETUS CORPORATION) 9 September 1987, see the whole document -----	1-36

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 89/00120**

SA 26896

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EPO file on 03/03/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 88/10315	29/12/88	NONE	
EP-A2- 0236069	09/09/87	AU-D- 69180/87 JP-A- 62240862	27/08/87 21/10/87

EPO FORM 1807  
For more details about this annex : see Official Journal of the European Patent Office, No. 12/82